HUMAN GENE THERAPY 13:841–853 (May 1, 2002)

Characterization of Lentiviral Vector-Mediated Gene Transfer in Adult Mouse Brain

VEERLE BAEKELANDT,[1] ANJE CLAEYS,[2] KRISTEL EGGERMONT,[1] ERWIN LAUWERS,[1] BART DE STROOPER,[3] BART NUTTIN,[1] and ZEGER DEBYSER[2]

ABSTRACT

Lentiviral vectors are promising tools for gene transfer into the central nervous system. We have characterized in detail transduction with human immunodeficiency virus type 1 (HIV-1)-derived vectors encoding enhanced green fluorescent protein (eGFP) in the adult mouse brain. Different brain regions such as the striatum, hippocampus, and the lateral ventricle were targeted. The eGFP protein was transported anterogradely in the nigrostriatal pathway, but we have found no evidence of transport of the lentiviral vector particle. The performance levels of the different generations of packaging and transfer plasmid were compared. Omission of the accessory genes from the packaging plasmid resulted in a modest decrease in transgene expression. Inclusion of the woodchuck hepatitis posttranscriptional regulatory element, on the one hand, and the central polypurine tract and termination sequences, on the other hand, in the transfer vector each resulted in a 4- to 5-fold increase in transgene expression levels. Combination of both elements enhanced expression levels more than the sum of the individual components, suggesting a synergistic effect. In the serum of mice injected with lentiviral vectors a humoral response to vector proteins was detected, but this did not compromise transgene expression. Immune response to the transgene was found only in a minority of the animals.

OVERVIEW SUMMARY

Gene therapeutic approaches for neurodegenerative diseases depend on the availability of a vehicle that can safely and efficiently introduce genes into neuronal cells. The lentiviral vectors (LVs) seem to fulfill most of the requirements for successful gene transfer into the central nervous system. This study provides an extensive characterization of HIV-1-derived lentiviral vectors in adult mouse brain. We have carefully monitored LV transduction with different generations of packaging and transfer plasmid, in different brain regions and at different time points. Aspects of neurotoxicity and immune response associated with LV transduction were also analyzed. Our work has implications for preclinical evaluation of HIV-1-derived vectors for gene therapy of central nervous system diseases and as a research tool for gene transfer into the brain.

INTRODUCTION

THE CONCEPT OF gene therapeutic approaches for diseases of the central nervous system (CNS) has come of age (Karpati *et al.*, 1996; Baekelandt *et al.*, 2000b). Potential applications include not only congenital single-gene disorders; support is growing to interfere at the genetic level for brain tumors and acquired chronic diseases that involve complex pathways and multiple genes. *In vivo* gene therapy for CNS disease depends on the availability of a vehicle that can efficiently, stably, and safely introduce therapeutic genes into differentiated neurons.

The lentiviral vectors (LVs) seem, next to adeno-associated viral vectors, to fulfill most of the requirements for successful gene transfer into the CNS (Amado and Chen, 1999). Indeed, they have proven efficient at providing long-term gene expression in rat brain (Naldini *et al.*, 1996; Blömer *et al.*, 1997) and in monkey brain (Kordower *et al.*, 1999), without any indica-

[1]Gene Therapy Program, Laboratory for Experimental Neurosurgery and Neuroanatomy, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium.
[2]Gene Therapy Program, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium.
[3]Gene Therapy Program, Center for Human Genetics, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium.

tion of loss of transgene expression or immune response. A preferential *in vivo* transduction of neurons over glial cells was observed but remains largely unexplained. LVs were also reported to successfully transduce primary neurons (cerebellar, hippocampal, and motor neurons) in culture (Mochizuki *et al.*, 1998; Mitrophanous *et al.*, 1999; Cisterni *et al.*, 2000). One of the major concerns for the applicability of lentiviral vectors for gene therapy is the issue of biosafety, which has guided improvements in lentiviral vector technology (Zufferey *et al.*, 1997, 1998; Dull *et al.*, 1998). Alternatively, vectors derived from lentiviruses other than human immunodeficiency virus type 1 (HIV-1) (feline immunodeficiency virus [FIV], equine infections anemia virus [EIAV], and simian immunodeficiency virus [SIV]) have been designed as well (Poeschla *et al.*, 1998; Mitrophanous *et al.*, 1999; White *et al.*, 1999), although it is still under debate whether these humanized vectors are any more safe than the HIV-derived vectors.

In animal models, proof of principle for a therapeutic effect after lentiviral vector-mediated gene transfer into the CNS has already been demonstrated. Blömer *et al.* (1998) showed that LV encoding antiapoptotic Bcl-xL or human nerve growth factor could protect adult septal cholinergic neurons from axotomized cell death. In the *rd* mouse, an animal model for retinitis pigmentosa, administration of the rod photoreceptor cGMP phosphodiesterase β subunit (PDEβ) gene into the subretinal space partially rescued the photoreceptor cells from degeneration in about 40% of the animals (Takahashi *et al.*, 1999). Reversal of brain damage in approximately the entire brain was obtained by using an HIV-1-based vector in the β-glucuronidase-deficient mucopolysaccharidosis type VII mouse, an animal model of human lysosomal storage diseases (Bosch *et al.*, 2000). Furthermore, LVs expressing glial cell-derived neurotrophic factor (GDNF) conferred neuroprotection in rodent and primate models of Parkinson's disease (Bensadoun *et al.*, 2000; Deglon *et al.*, 2000; Kordower *et al.*, 2000; Rosenblad *et al.*, 2000) and amyotrophic lateral sclerosis (ALS) (Hottinger *et al.*, 2000). Finally, correction of neuropathology and learning impairments was obtained in a mouse model of metachromatic leukodystrophy (Consiglio *et al.*, 2001).

Despite these early, promising reports for potential clinical applications, some important issues of LV-mediated transduction with implications for using the vectors in gene therapy or as research tool for CNS gene transfer have not been addressed in detail. We have used HIV-1-derived LVs (Naldini *et al.*, 1996) carrying the marker gene encoding enhanced green fluorescent protein (eGFP) for a thorough characterization of *in vivo* gene transfer into the adult mouse brain. We report here on the performance of different generations of vector, on the time course of transgene expression, on target cell specificity, and on the immune response to and toxicity of LV-mediated gene transfer.

MATERIALS AND METHODS

Lentiviral vector production

HIV-1-derived vector particles, pseudotyped with the envelope of vesicular stomatitis virus (VSV), were produced by transfecting 293T cells with a packaging plasmid encoding viral Gag, Pol, and accessory proteins (pCMVΔR8.2), a plasmid encoding the envelope of vesicular stomatitis virus (pMDG), and a plasmid encoding a reporter gene flanked by two long terminal repeats (LTRs) (pHR'-CMVeGFP) (Naldini *et al.*, 1996). A second-generation attenuated packaging plasmid (pCMVΔR8.91) that lacks *vif*, *vpr*, *vpu*, and *nef* genes (Zufferey *et al.*, 1997) and a transfer plasmid with a self-inactivating deletion in U3 (pHR'SIN-18) (Miyoshi et al., 1998; Zufferey *et al.*, 1998) were used as well. The integrase gene in the packaging plasmid pCMVΔR8.2ΔIN contains the D64V mutation that renders the integrase protein catalytically inactive (Cherepanov *et al.*, 2000). All plasmids were kindly provided by O. Danos (Evry, France) and D. Trono (Geneva, Switzerland), whereas pHR'TRIPGFP and pHR'TRIPinvGFP, containing the central polypurine tract (cPPT) and central termination sequences (CTS), were kindly provided by P. Charneau (Institut Pasteur, Paris, France). A transfer plasmid containing a multiple cloning site, the SIN-18 deletion, the woodchuck hepatitis posttranscriptional regulatory element (WPRE), and the cPPT/CTS sequence was created in house and named *combivector*. For transfection of a 10-cm dish of 293T cells, 20 μg of vector plasmid, 10 μg of packaging construct, and 5 μg of envelope plasmid were mixed in 700 μl of 150 m*M* NaCl. A volume of 700 μl of polyethyleneimine (PEI) solution (1.57 m*M* PEI in 150 m*M* NaCl) was added slowly. The mixture was incubated at room temperature for 15 min and then added dropwise to the 293T cells in Dulbecco's modified Eagle's medium (DMEM) with 1% fetal calf serum (FCS). The next morning, medium was replaced with DMEM containing 10% FCS. Supernatants were collected from day 2 and filtered until day 5 posttransfection. The vector particles in the supernatant were sedimented by ultracentrifugation in a swinging-bucket rotor (SW27; Beckman, Palo Alto, CA) at 25,000 rpm for 2 hr at 4°C. Pellets were redissolved in 300 μl of phosphate-buffered saline (PBS), resulting in a 100-fold concentration. p24 antigen content was determined by HIV-1 p24 Core Profile enzyme-linked immunosorbent assay (ELISA) (DuPont, Dreieich, Germany). p24 content was used to normalize the different vector batches. Titers for vectors encoding eGFP were determined in CHO cells by fluorescence-activated cell sorting (FACS) analysis.

Surgery

Adult C57BL/6 and CB-17/*scid* × *scid* mice of both sexes were used. The animal experiments were approved by the bioethics committee of the Katholieke Universiteit Leuven (Leuven, Belgium). All surgical procedures were performed under chloral hydrate anesthesia (400 mg/kg, intraperitoneal), using aseptic procedures. The mice were placed in a stereotactic head frame, and after midline incision of the skin, one or two small holes were drilled in the skull in the appropriate location, using bregma as reference (Franklin and Paxinos, 1997). Coordinates used were as follows: striatum (anteroposterior [AP], 0.5; lateral [LAT], 2.0; dorsoventral [DV], 3.0–2.0), hippocampus (target 1: AP, −3.16; LAT, 3.0; DV, 3.0–2.0; target 2: AP, −2.3; LAT, 2.0; DV, 1.5–1.25), lateral ventricle (AP, −0.46, LAT, 1.00; DV, 1.80). Two microliters of highly concentrated vector (10^8 pg of p24 per milliliter) supplemented with polybrene (4 μg/ml) was injected at a rate of 0.5 μl/min with a 30-gauge needle on a 10-μl Hamilton syringe. In some animals, 2 μl of 0.9% NaCl solution was injected into the contralateral hemisphere as a control. For the intraventricular in-

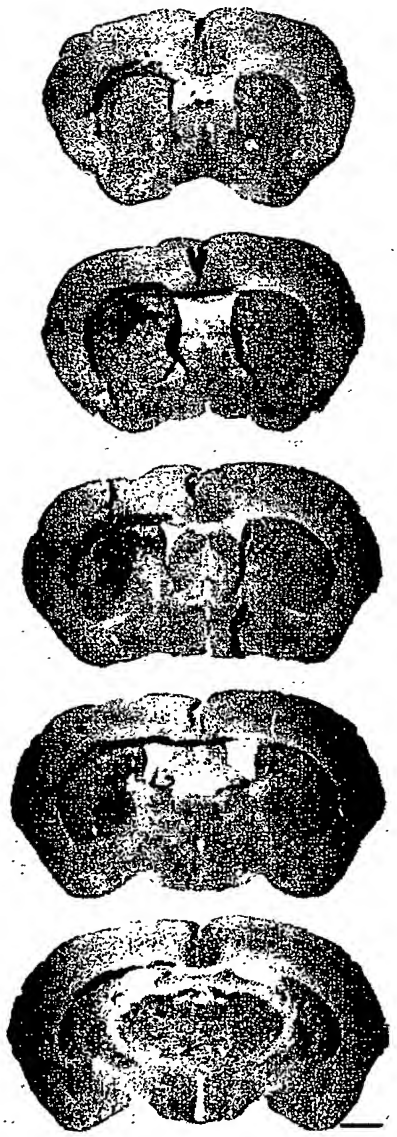

FIG. 1. LV transduction of the mouse striatum. Serial sections with an interval of 500 μm through the striatum of a C57BL mouse 2 weeks after transduction with a first-generation LV encoding eGFP. eGFP expression was visualized immunohistochemically. Scale bar: 1 mm.

jections, 4 μl of vector was used. During the 4 min of injection, the needle was raised slowly in the dorsal direction over the distance indicated by the two DV coordinates. After the injection, the needle was left in place for an additional 4 min before being slowly withdrawn from the brain (adapted from Dull *et al.*, 1998).

For the boost experiment a separate group of animals received injections into the left striatum. At 2 months some of the animals received injections into the right striatum. Two weeks later, the animals were killed.

Histology

To assess lentiviral transduction, the mice were deeply anesthetized with pentobarbital and perfused transcardially with saline followed by ice-cold 4% paraformaldehyde in PBS for 15 min. The brain was removed from the skull and postfixed overnight in the same fixing solution. Coronal brain sections (50 μm thick) were cut with a Vibratome and stored at 4°C in PBS buffer containing 0.1% sodium azide. The sections were first screened for eGFP protein expression with an inverted fluorescence microscope, but more sensitive detection was obtained with immunohistochemistry, using a polyclonal antibody against eGFP (Clontech, Palo Alto, CA). The sections were treated with 3% hydrogen peroxide and preincubated in 5% normal swine serum with 0.1% Triton X-100 in PBS. Incubation with the primary rabbit anti-GFP antibody (diluted 1:1000) in 5% normal swine serum and 0.1% Triton X-100 was done overnight at room temperature. The sections were then incubated in biotinylated swine anti-rabbit secondary antibody, followed by an incubation with Strept-ABC-HRP complex (Dako, Glostrup, Denmark). Detection was with diaminobenzidine (DAB), using H_2O_2 as a substrate. Identification of astrocytes and neurons was based on staining with a polyclonal antibody

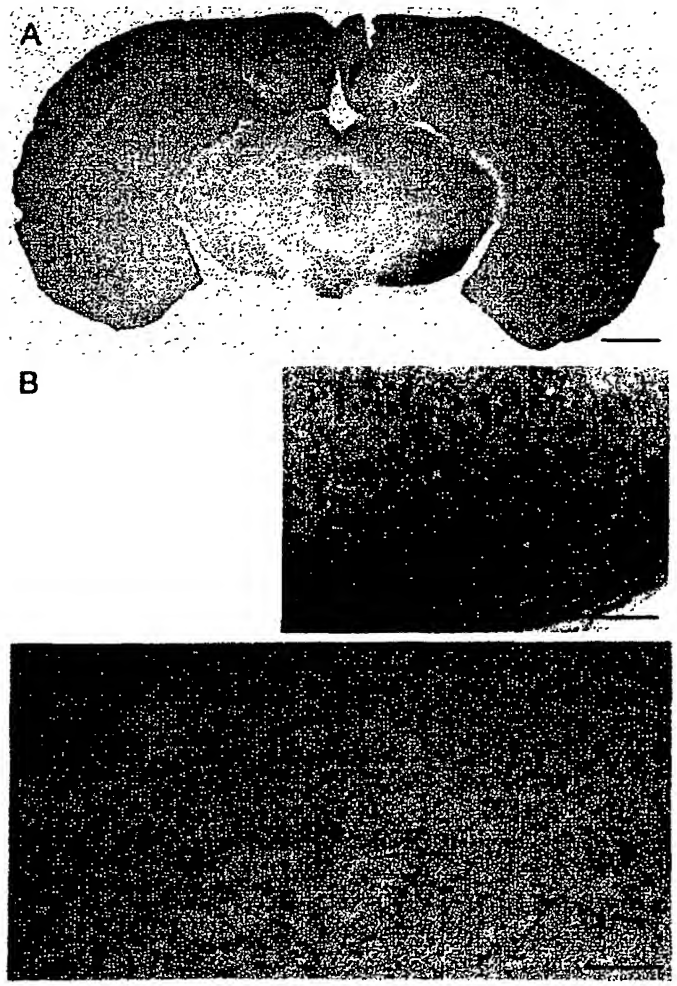

FIG. 2. Anterograde transport of the eGFP protein after LV transduction in the nigrostriatal pathway. (A and B) eGFP protein expression in the substantia nigra 2 weeks after injection of a second-generation LV containing the WPRE into the striatum. (A) Overview. (B) Higher magnification shows eGFP-positive fibers in the substantia nigra pars reticulata. (C) eGFP-positive fibers in the striatum after injection of LV into the substantia nigra. Scale bars: (A) 1 mm; (B and C) 100 μm.

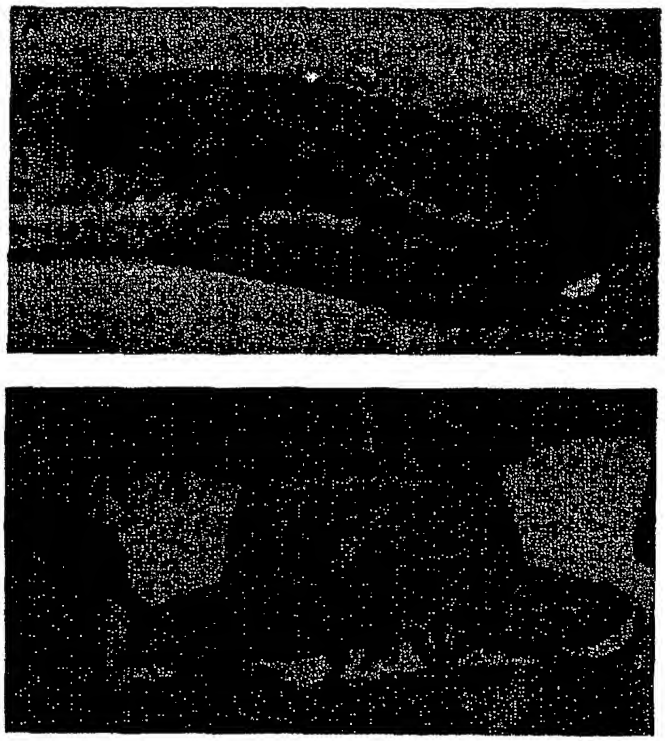

FIG. 3. LV transduction of the mouse hippocampus and lateral ventricle. eGFP expression 2 weeks after transduction with a second-generation LV in the hippocampus (A) and in the lateral ventricle (B) of a C57BL mouse. Scale bars: 100 μm.

against glial fibrillary acidic protein (GFAP; Dako) and a monoclonal antibody against NeuN (Chemicon, Temecula, CA), respectively. To detect macrophages, we used a monoclonal antibody against mouse CD11b (Serotec, Oxford, UK). T lymphocytes were stained with monoclonal antibodies against mouse CD4 and CD8 (BD Pharmingen, San Diego, CA).

Apoptosis

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining was performed according to a protocol adapted from Young *et al.* (1999). Briefly, sections are pretreated with 1% H_2O_2 in methanol, followed by an incubation in 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. The labeling reaction with terminal deoxynucleotidyltransferase (TdT) in the presence of biotin 16-dUTP is for 4 hr at 37°C. The sections are then incubated with Strept-ABC-HRP complex (Dako) and stained with DAB and H_2O_2. As a positive control, sections are treated with DNase I for 30 min at 37°C before the TdT incubation.

Quantification of gene expression

The volume of the transduced brain area was quantified by a stereological procedure based on the Cavalieri principle (Sterio, 1984). For each animal serial sections (minimum of six) with an interval of 500 μm centered around the injection site were analyzed by means of a Bioquant image analyzing system (R&M Biometrics, Nashville, TN) connected with a CCD video camera to the microscope. A point-counting grid was placed over the screen on which the entire transduced brain region was displayed from a low-power objective. Points overlying eGFP-positive cells and fibers were counted. The transduced volume was calculated by multiplying the sum of the counted points with the distance between the counted sections and the area associated with each point on the grid.

Immune response

Before perfusion, a blood sample was taken from the heart of each mouse. Blood sera were analyzed for antibodies directed against the transgene and against the LV preparation by Western blot. Briefly, 20 ng of purified eGFP was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto nitrocellulose membrane, followed by incubation with individual mouse sera diluted 1:500. Detection was by subsequent incubation with an HRP-labeled secondary antibody and development with an ECL$^+$ kit (Amersham Pharmacia, Uppsala, Sweden). As a negative control, we used sera from unoperated mice, from mice injected with eGFP PEI, and from mice injected with LVs encoding a gene unrelated to GFP (amyloid precursor protein). As a positive control, we used two rabbit polyclonal antisera to eGFP (one antiserum from Clontech and one in-house antiserum). Immune response to the lentiviral vector proteins was examined by blotting HIV-1-derived vector encoding β-galactosidase (10 ng of p24 per lane). Unoperated mice and mice injected with eGFP PEI were used as a negative control. HIV-1-specific bands were identified by comparison with serum from an HIV-1-positive patient.

RESULTS

LV transduction in different areas of the adult mouse brain

Intraparenchymal injection of HIV-1-derived LVs (typically 10^5 pg of p24) encoding eGFP (LV-eGFP) into the adult mouse brain resulted in high-level expression of the transgene. Striatal delivery of LVs transduced neural cells in the striatum and in the overlying corpus callosum and cortex over a large brain volume (Fig. 1). In addition, we observed eGFP-positive fibers in the substantia nigra pars reticulata of the injected hemisphere (Fig. 2), implying that the eGFP protein was transported anterogradely along the striatal efferent pathways. Concordantly, we also found eGFP-positive fibers in the striatum of mice and rats receiving injections into the substantia nigra (Fig. 2). We never observed eGFP-positive cells in the substantia nigra pars compacta after transduction of the striatum. In hippocampus, transduction was also successful, but the transduced brain volume was more variable than in striatum (Fig. 3A). Because of the anatomical organization of the hippocampus the vector can probably spread along the folds of Ammon's horn. LV-eGFP injected into the lateral ventricle, on the other hand, transduced only ependymal cells lining the ventricle and choroid plexus cells, and not the adjacent brain parenchyma (Fig. 3B).

Comparison of different generations of LVs

We have compared the performance of different constructs and preparations of HIV-1 derived LVs encoding eGFP. The

TABLE 1. CHARACTERISTIC PARAMETERS FOR THE DIFFERENT VECTOR PREPARATIONS

	Relative p24[a]		*Relative TU/pg*[b]	
Vector	*8.2*	*8.91*	*8.2*	*8.91*
pHR'	1.00	1.00	1.00	1.00
pHR'SIN-18	0.7 ± 0.1	0.30 ± 0.06	0.16 ± 0.01	0.36 ± 0.12
pHR'WPRE	0.89 ± 0.20	0.26 ± 0.07	1.7 ± 0.8	7.80 ± 1.15
pHR'TRIP	0.87 ± 0.29	0.65 ± 0.14	4.6 ± 1.0	42.0 ± 6.1
pHR'TRIPinv	1.30 ± 0.36	0.70 ± 0.21	0.99 ± 0.29	2.5 ± 0.6

[a]p24 values were determined for parallel, small-scale vector productions ($n = 2$ or 3) with different combinations of transfer and packaging plasmids. Average values ± SE are presented relative to the p24 values obtained with pHR' as transfer plasmid and 8.2 or 8.91 as packaging plasmid. All vectors encode eGFP. The p24 value for pHR' with 8.91 relative to pHR' using 8.2 was 0.81.

[b]The different vectors produced were titered on CHO cells. After 3 days titers were determined by FACS analysis based on end-point dilution (transduction units per milliliter, or TU/ml). Titers were normalized for p24 concentration (TU/pg). Results presented are average values ± SE for two or three titrations and are presented relative to values obtained for pHR'. The absolute TU/pg values for pHR' were 9.4 ± 2.1 and 3.8 ± 1.3 using 8.2 or 8.91, respectively.

production characteristics, the p24 content, and the transducing titer on CHO cells were compared for various preparations (Table 1). The first-generation packaging plasmid 8.2 contains four accessory genes that were removed in 8.91 (Zufferey *et al.*, 1997). When using 8.91 a small decrease (20%) in p24 yield was observed and the transducing titers were 2-fold lower. Introduction of the self-inactivating deletion in the 3' U3 (SIN-18) (Zufferey *et al.*, 1998), resulted in lower vector yield and titer. Introduction of the woodchuck hepatitis posttranscriptional regulatory element (WPRE) is reported to enhance transgene expression (Zufferey *et al.*, 1999). We observed a 4-fold decrease in p24 level when using 8.91 but an 8-fold increase in apparent titer. The cPPT (TRIP) has been reported to increase transduction efficiency of HIV-1 vectors, an effect attributed to improved nuclear import of the preintegration complex (Follenzi *et al.*, 2000; Zennou *et al.*, 2000). The titers of cPPT containing vectors were 5- to 40-fold higher than for vectors without cPPT produced in parallel. The orientation of cPPT was essential; in inverse orientation there was a much smaller effect on transduction efficiency, as has been reported (Zennou *et al.*, 2000).

Next, the transduction efficiency was measured for the different vector preparations 2 weeks after gene delivery in mouse striatum (Table 2). Expression was verified immunohistochemically and quantified by stereological transduced volume measurements. Vectors made with the second-generation packaging plasmid (8.91) were 2-fold less efficient than those produced with 8.2. Still, 8.91 in combination with the self-inactivating (SIN) transfer vector was selected for further experiments because of the improved biosafety profile (Zufferey *et al.*, 1997). Insertion of the WPRE into the transfer plasmid resulted in about 4-fold higher expression levels of eGFP. The presence of the cPPT increased expression levels about 5-fold. The inverse cPPT served as a control (data not shown). Insertion of both WPRE and cPPT sequence into the combivector further enhanced expression levels about 25-fold compared with the first-generation vector. To assure that our measurements were performed in a linear dose–response range, we have injected several dilutions of vectors. At a p24 concentration of about 10^7 pg/ml, saturation of eGFP expression levels was only reached with the combivector. The Tat-independent generation of packaging plasmid has not been tested (Dull *et al.*, 1998). The different plasmids used for lentiviral vector production were purified from bacterial endotoxins, which resulted in much more reproducible vector titers, but no significant differences in transduction efficiency *in vivo* (data not shown). For *in vivo* transduction, the LV preparation routinely contains the polycation polybrene. Substitution of poly-

TABLE 2. TRANSDUCTION OF MOUSE BRAIN WITH DIFFERENT VECTOR PREPARATIONS

Transfer vector	*Packaging plasmid*	*Relative eGFP*[a] *expression*
pHR'	8.2	1.00 ± 0.1
	8.91	0.45 ± 0.02
pHR'SIN-18	8.91	0.5 ± 0.1
pHR'WPRE	8.91	3.90 ± 0.03
pHR'TRIP	8.91	5.00 ± 0.01
pHR'COMBI	8.91	27.6[b] ± 8.2

[a]eGFP expression was measured immunohistochemically 2 weeks after injection of different vector preparations. Quantification of expression is explained in Materials and Methods. Average values ± SE for two to five mice are shown. Results were normalized for p24 content and are presented relative to the expression obtained with a vector made with pHR' and p8.2 (1 corresponds to 15 $mm^3/\mu g$ of p24).

[b]These values result from injections of p24 concentrations of about 10^7 pg/ml, as used for the other vector preparations. At p24 concentrations of about 10^5 pg/ml the values of the combivector were even 10-fold higher, suggesting a saturation of transduction at the highest concentration used.

brene by the lipopolyamine dioctadecylamidoglycylspermine (DOGS) (Themis *et al.*, 1998) did not increase transduction efficiency (data not shown).

Time course of transgene expression

We have examined expression of eGFP at different time points after injection. At 2 days, eGFP-positive cells were already visible adjacent to the needle track, but the expression levels were considerably lower than at 2 weeks (Fig. 4). At the 2-week time point, transgene expression reached a maximum level that appeared stable for at least 2 months. So far, we have analyzed two mice 1 year after transduction. In both cases, the animals displayed prominent eGFP transgene expression levels similar to the level at 2 months.

Identification of transduced cells

The LVs transduced cells of both neuronal and glial morphology in the adult mouse brain. This was confirmed by double stainings and confocal microscopy (Fig. 5). The proportion of neurons to nonneuronal cells was quantified by counting cells double labeled with GFAP or NeuN in five animals at time points between 2 weeks and 1 year after transduction. The proportion of neuronal cells varied from 45 to 80%, with a trend for a higher proportion of neuronal cells at later time points. In contrast, eGFP-expressing cells 2 days after injection were predominantly nonneuronal. Expression levels in neurons were generally lower than in astrocytes. Interestingly, LVs containing a defective integrase protein (D64V integrase) also transduced primarily cells with glial morphology (about 75%, versus 25% neuronal) (Fig. 6).

Immune response to vector and transgene

Possible interference of the host immune system with transgene expression was examined by comparing LV transduction in immune-competent C57BL mice and in severe combined immunodeficiency (*scid*) mice. There was no difference in magnitude or stability of transgene expression between the two mouse strains (Baekelandt *et al.*, 2000a). A possible humoral response to vector and transgene proteins was examined by analysis of the sera of the injected mice. Immunoreactivity to various vector proteins was observed in all immune-competent mice after LV transduction (Fig. 7A). Comparison with the antibodies present in the serum of a human HIV-positive subject allowed us to relate some bands to HIV proteins. Nevertheless, reinjection of LV-eGFP into the second hemisphere 2 months after the first injection was successful in C57BL and *scid* mice with equivalent expression levels of the transgene (Fig. 9). Antibody response to the eGFP transgene protein was weak and was detected only in a minority of the animals (Fig. 7B).

Toxicity and pathology associated with LV transduction

The general cytoarchitecture, visualized by Nissl stains, displayed remarkably little pathology in the transduced brain area. We observed cell debris and occasional local bleeding in the needle track only at the early time points examined (2 days and

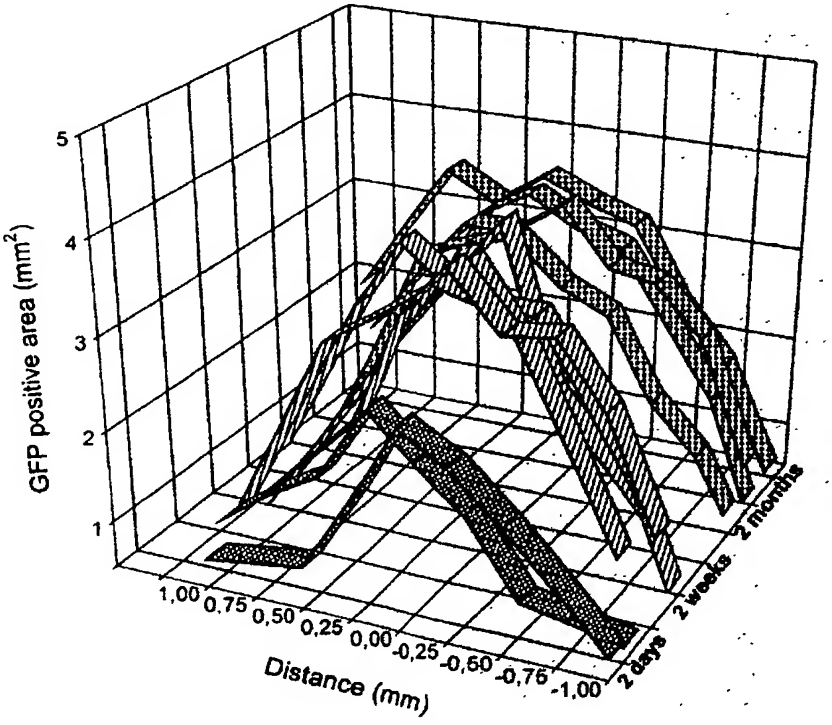

FIG. 4. Time course of transgene expression. Shown is the quantification of eGFP expression in the striatum on serial sections over a distance of 2 mm around the injection site 2 days ($n = 2$), 2 weeks ($n = 3$), and 2 months ($n = 3$) after lentiviral transduction.

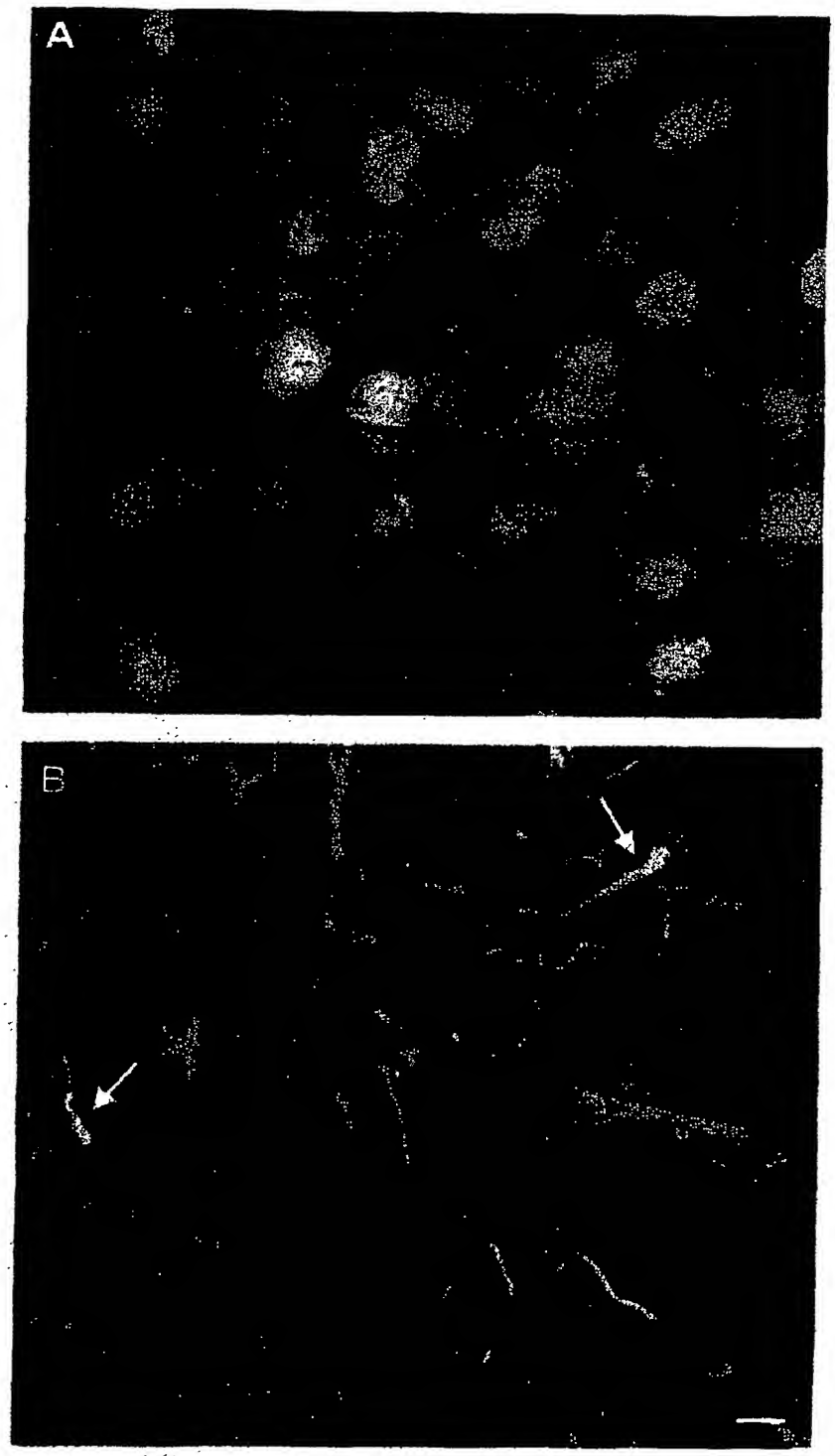

FIG. 5. LVs transduce neuronal and glial cells. (A) Colocalization by confocal microscopy of the neuronal marker NeuN (red) with eGFP-positive cells 2 months after transduction with a second-generation LV containing the WPRE. (B) Staining with the astrocytic marker GFAP (red) in the same mouse shows a few eGFP-positive glial cells (arrows). Scale bar: 10 μm.

2 weeks). At longer survival times, the damage was indistinguishable from the mechanical damage caused by saline control injection. In a minority of animals (<10%), CD11b-positive macrophages were found in the needle track and adjacent corpus callosum, usually at short time points after injection. Similarly, a limited number of CD4- and CD8-positive T lymphocytes was occasionally detected in close proximity to the injection site (data not shown). GFAP stainings, on the other hand, showed a rapid and extensive proliferation of reactive astrocytes in approximately the whole ipsilateral hemisphere after LV transduction (Fig. 8). This reaction was also observed with control saline injections, but to a lesser extent. The as-

FIG. 6. Transduction with a LV containing a deficient integrase protein. (A) eGFP expression 2 weeks after transduction of the left mouse striatum with a first-generation pHR'-WPRE LV containing the D64V integrase mutation, and of the right striatum with a control pHR'-WPRE LV. (B) Higher magnification view of the left hemisphere. (C) Higher magnification view of the right hemisphere. Scale bars: (A) 1 mm; (B and C) 100 μm.

trogliosis subsided gradually and was restricted to the needle track by 6 months after injection.

To our surprise, LV transduction resulted in a transient decrease in NeuN immunoreactivity in the ipsilateral hemisphere. The area where loss of NeuN expression was evident often exceeded the area of transgene expression (Fig. 9). The decrease in NeuN immunoreactivity cannot be explained by a loss of neurons in the affected area because Nissl stain did not show any cell loss. Neither did we find any evidence of apoptotic cell death. TUNEL staining 2 days and 2 weeks after LV transduction was always negative (Fig. 10). Furthermore, at longer survival times NeuN expression returned to normal in the transduced brain area (Fig. 9). The transient loss of NeuN immunoreactivity was observed in mouse and rat brain, but not in the immune-deficient *scid* mice.

DISCUSSION

Lentivirus and adeno-associated virus vectors are currently regarded as best choice tools for gene transfer and gene therapy in the CNS (Amado and Chen, 1999; Baekelandt *et al.*, 2000b). The performance of LVs is superior to that of adenoviral vectors with regard to stability of gene expression and absence of inflammatory response. In comparison with the widely used retroviral vectors, LVs offer the advantage of transduction of nondividing cells. With this characterization study, we wanted to carry out an independent evaluation of LV technology. Overall, our study confirmed the ability of HIV-1-derived LVs to transduce neuronal cells *in vivo* efficiently. Particular details of our study, however, revealed new and interesting information and even some discrepancies with published data.

In concordance with other publications (Blömer *et al.*, 1997; Kordower *et al.*, 1999; Bensadoun *et al.*, 2000; Deglon *et al.*, 2000), we have obtained high expression levels of the eGFP marker gene in different regions of the mouse brain. In contrast to results with adeno-associated viral vectors, no differences in transduction efficiency between different brain regions were found (McCown *et al.*, 1996; Klein *et al.*, 1998; Kirik *et al.*, 2000). In the hippocampus there was a preferential transduction of cells in the hilus and at the border with the dentate granule cell layer, sites that correspond to the location of neural progenitor cells (Kuhn *et al.*, 1996). Further experiments are needed to identify the exact nature of the transduced hippocampal cells. The cell type-specific expression might be due to the cytomegalovirus (CMV) promoter as suggested by Smith *et al.* (2000). The presence of eGFP-positive fibers in the substantia nigra pars reticulata after LV transduction in the striatum, and in the striatum after transduction of the substantia nigra pars compacta, indicates that eGFP was transported anterogradely over considerable distances in the nigrostriatal pathway. Anterograde transport of the protein encoded by the transgene has been described elsewhere for GDNF (Kordower *et al.*, 2000) and for GFP with adeno-associated (Kirik *et al.*, 2000) and lentiviral vectors (Rosenblad *et al.*, 2000). In contrast to an initial report from Blömer *et al.* (1997), we and others (Kordower *et al.*, 1999, 2000) found no evidence of retrograde transport of the lentiviral vector itself, as we never

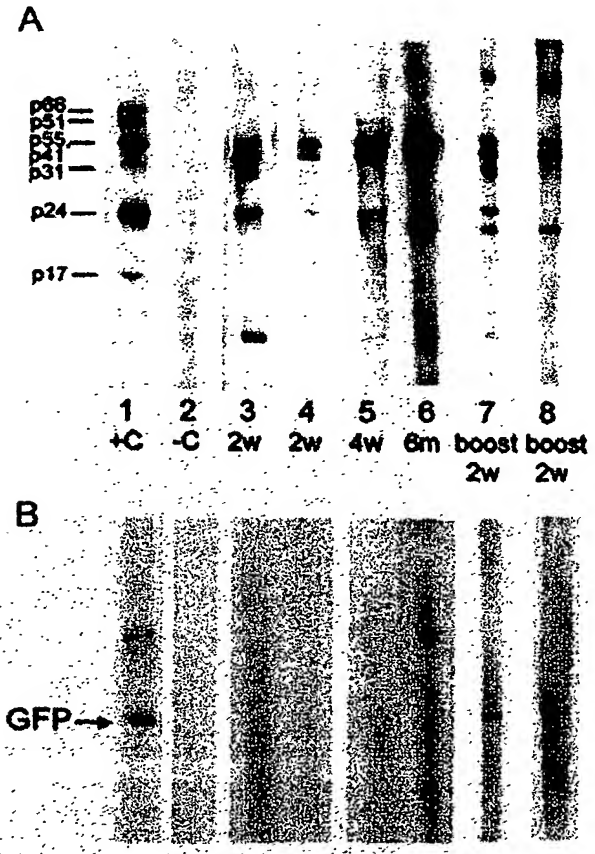

FIG. 7. Detection of serum antibodies after LV transduction. Shown is an analysis by Western blot of the immune response to LV proteins (A) and to the eGFP transgene (B) after LV transduction. (A) LV proteins were separated by SDS–PAGE and incubated with sera from mice transduced with LV encoding eGFP obtained at different time intervals after injection (2w = 2 weeks; 4w = 4 weeks; 6m = 6 months). Serum from an HIV-positive patient was used as a positive control (+C). Serum from a mouse injected with saline served as a negative control (−C). The mice in the boost experiment were first injected in the left hemisphere, 2 months later in the other hemisphere, and killed 2 weeks after the second injection. (B) Purified eGFP protein was separated by SDS–PAGE and incubated with sera from mice injected with LV encoding eGFP at different time points [see (A)]. Positive control is a polyclonal rabbit antibody to eGFP. Serum from a mouse injected with a LV encoding a nonrelated protein was used as a negative control.

observed eGFP-positive somata in the substantia nigra pars compacta after transduction in the striatum.

We have carefully compared the *in vivo* performance of different generations of LVs in the mouse brain. The subsequent generations of vectors were designed to increase biosafety with a minimal loss in efficiency (Zufferey *et al.*, 1997). In our hands, use of the second-generation packaging plasmid resulted in a modest decrease in expression levels of the transgene. Zufferey *et al.* reported equal efficiency of the first- and second-generation plasmids for *in vivo* gene delivery into the rat brain, but described that HIV-mediated gene delivery to terminally differentiated macrophages was reduced by approximately 50% in the absence of *vpr* (Zufferey *et al.*, 1997). Kafri *et al.* reported a substantial reduction in transduction of liver by use of the second-generation packaging plasmid, but an undiminished and perhaps even enhanced transduction efficiency in neurons in the brain (Kafri *et al.*, 1997). Which of the accessory genes *vpr*, *vif*, *nef*, or *vpu* is responsible for the observed reduction in transduction is not clear at this moment. The U3 deletion (SIN-18) did not hamper transgene expression and was included for biosafety reasons. Insertion of the WPRE and of the cPPT sequence in the transfer vector each increased eGFP expression levels about 4- and 5-fold, respectively, in accord with the data from cell culture (Zufferey *et al.*, 1999; Zennou *et al.*, 2000) and *in vivo* (Deglon *et al.*, 2000; Follenzi *et al.*, 2000; Zennou *et al.*, 2001). On the basis of these results we decided to develop, for gene transfer in the brain, a standard vector containing both the cPPT and WPRE in combination with the SIN deletion (combivector). The *in vivo* transduction efficiency of this combivector turned out to be greater than the sum of the ex-

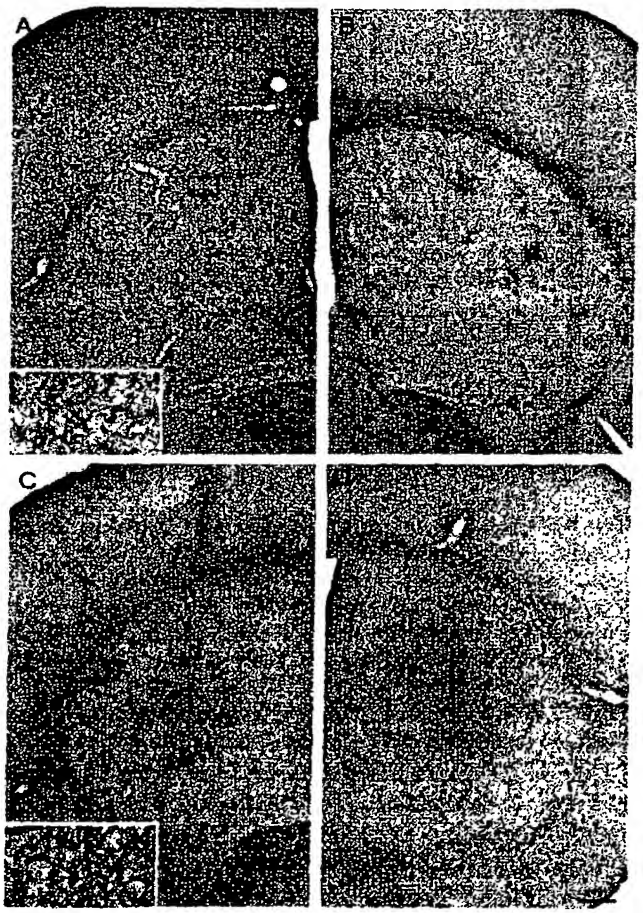

FIG. 8. Transient proliferation of reactive astrocytes after lentiviral transduction. Immunostaining for GFAP shows numerous astrocytes in the entire hemisphere 2 weeks after transduction with a first-generation LV. Inset in (A) shows a higher magnification of the area marked with a black box. (B) GFAP immunostaining in a hemisphere that was not injected. (C) Immunostaining for GFAP 2.5 months after lentiviral transduction shows reactive astrocytes, mainly around the needle track. (D) Immunostaining for GFAP 2 weeks after injection with saline solution. Scale bars: 1 mm; inset, 100 μm.

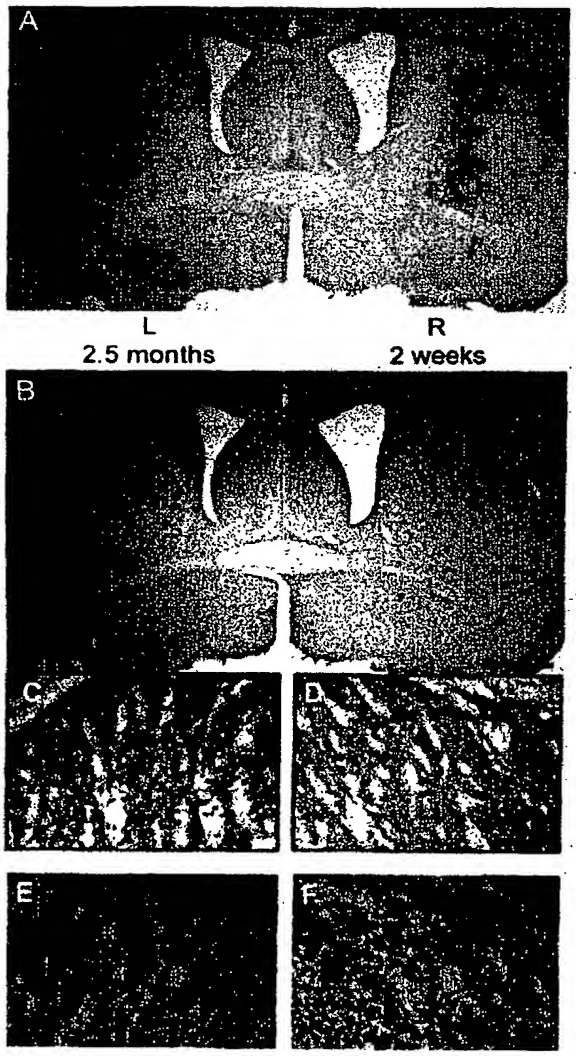

FIG. 9. Lentiviral transduction induces a transient decrease in NeuN immunoreactivity. (A) Immunostaining for eGFP 2 weeks (right hemisphere) and 2.5 months (left hemisphere) after lentiviral transduction with a first-generation LV. (B–D) Immunostaining for NeuN in an adjacent section shows normal expression in the left hemisphere (B and C) and an almost complete absence of immunoreactivity in the right hemisphere (B and D). (E and F) Cresyl violet staining in an adjacent section shows no significant differences between the left (E) and right (F) hemisphere. Scale bars: (A and B) 1 mm; (C–F) 100 μm.

pression levels from the cPPT and WPRE separately. A similar, synergistic effect of the cPPT and WPRE has been described in cell culture (Barry *et al.*, 2001). Long-term expression studies of marker genes with the combivector are underway. We plan to verify whether addition of one of the accessory genes to the packaging plasmid 8.91 can further improve expression levels. The unexpectedly high transgene expression levels obtained with the combivector will help to achieve sufficient amounts of therapeutic protein in the brain. On the other hand, it will also allow a considerable reduction in the amount of vector particles per injection, which may avoid problems of neurotoxicity and immune response.

The clear increase in eGFP expression between day 2 and day 14 precludes pseudotransduction by eGFP protein present in the LV preparation. In agreement with other reports (Blömer *et al.*, 1997; Kordower *et al.*, 1999; Deglon *et al.*, 2000; Kirik *et al.*, 2000), we have observed stable eGFP transgene expression for at least 1 year. To fully assess the requirement for insertion into the cellular genome, long-term expression levels after transduction with a LV containing a defective integrase enzyme should be determined. Several studies have reported that LVs transduce predominantly neurons (Blömer *et al.*, 1997; Kordower *et al.*, 1999). Other groups observed a significant

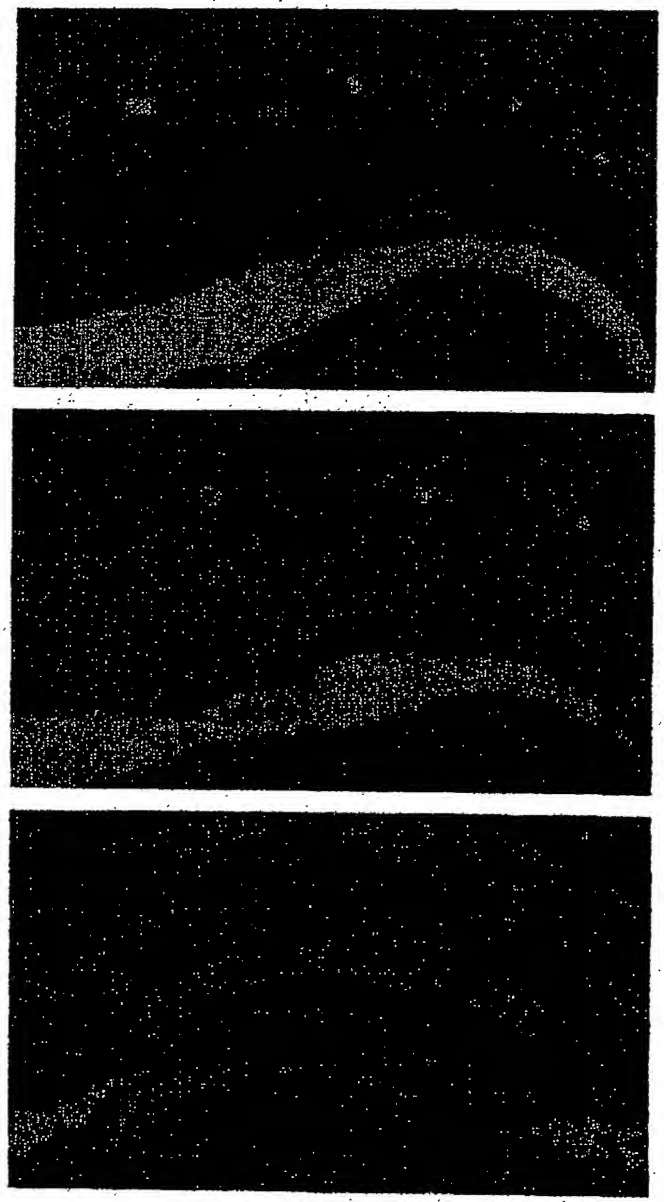

FIG. 10. No evidence of apoptosis after lentiviral transduction. (A) eGFP expression in the hippocampus 4 weeks after lentiviral transduction with a second-generation LV. (B) TUNEL staining of an adjacent section shows no apoptotic cells in the area of the hippocampus positive for eGFP expression. (C) DNase treatment of a section of the same mouse as positive control results in numerous labeled nuclei. Scale bar: 100 μm.

proportion of transduced astrocytes (Mitrophanous *et al.*, 1999; Bensadoun *et al.*, 2000). In our hands, LVs transduced between 50 and 80% of neuronal cells in the mouse brain. Glial morphology of eGFP-expressing cells was most prominent at early time points after transduction and, surprisingly, with LVs carrying an inactivating mutation in the integrase gene. This might suggest that expression from astrocytes is less dependent on the integration step. Although there was no difference in expression levels between 2 and 5 weeks after injection (data not shown), long-term experiments are underway to find out whether expression with an integrase-defective vector is maintained over time. The apparent shift in transduced cell type with time might be explained by the lower metabolic activity of mature neurons in the brain and hence the longer time lapse needed for integration and expression of the transgene, together with the gradual accumulation of the eGFP protein.

The excellent performance of LVs should eventually lead to clinical applications. One of the reasons for the interest in LVs for gene therapeutic applications is the apparent absence of immune response from the host, especially compared with adenoviral or herpesvirus-based vectors (Blömer *et al.*, 1997). We have seen no difference in transduction between immune-competent mice and *scid* mice. We have also performed successful readministration of LVs in the mouse brain without loss of transgene expression. Likewise, efficient transduction of hind-leg muscle of adult Fischer rats after a previous injection into brain or contralateral hind-leg muscle has been reported (Kafri *et al.*, 1997). However, our direct analysis of the sera of injected mice revealed the presence of circulating antibodies to vector proteins. Antibody response to the transgene was weak and was detected only in the case of a second injection with LVs. The humoral response in mice to vector proteins can probably be explained by the disruption of the blood–brain barrier during the stereotactic operation. It is not surprising that the current HIV vector preparations induce serum antibodies. Future clinical trials will need to examine patients for HIV-directed antibodies. Long-term expression of the transgene did not stimulate the immune system. Because no correlation was found between immune response and expression time or level, the antibody response to eGFP in the boost experiment is possibly due to the presence of eGFP protein as a contaminant in the vector preparation. Still, these eGFP antibodies do not seem to interfere with transgene expression. Experiments investigating the humoral response after LV transduction in more detail are ongoing in our laboratory.

Apart from gene therapeutic applications, LV technology can be used as a tool for basic research in neuroscience. LVs offer the possibility to analyze the effect of long-term local overexpression of specific genes. However, whereas high-level expression of eGFP can be obtained without the WPRE, expression of short-living proteins (eGFP has a half-life of 24 hr) requires upregulation of gene expression (our unpublished observations). With some genes, the presence of the WPRE is required to detect transgene expression. Before analyzing expression of disease-associated genes in mouse brain, a thorough characterization of the impact of LV transduction on neuronal cell function was required. First, we have observed reactive astrocytosis in the whole ipsilateral hemisphere. This reaction was more pronounced than after injection of saline and subsided slowly over time. Furthermore, our investigations have revealed that LV transduction induces transient alterations in normal neuronal gene expression. A transient but significant decrease in NeuN immunoreactivity was observed after lentiviral vector transduction. At longer survival times, both Nissl- and NeuN-stained sections revealed normal cytoarchitecture without any obvious neuron loss. NeuN is a DNA-binding neuron-specific nuclear protein present in most neuronal cell types of vertebrates (Mullen *et al.*, 1992). It is frequently used as a specific marker for differentiated neurons. NeuN immunoreactivity first appears at developmental time points that correspond with the withdrawal of the neuron from the cell cycle and/or with the initiation of terminal differentiation of the neuron. The exact function of the protein is still unknown, but it is believed to represent a nervous system-specific nuclear regulatory molecule. The decrease in NeuN immunoreactivity is possibly an indirect, immune system-mediated effect after LV transduction, as it was never observed in the immune-deficient *scid* mice.

The observed immune reactivity and the impact on neuron function after lentiviral transduction will require careful monitoring during early (pre)clinical trials. It would be interesting to verify whether both phenomena are restricted to rodents or can be detected in nonhuman primates as well. We are currently investigating whether further purification of the vector preparation can avoid both problems. Because alterations in neuronal function are transient, for gene expression analysis of disease-associated genes time points beyond 2 months after injection need to be included as well as control injections of a marker gene such as eGFP.

In conclusion, our study supports the view that lentiviral vectors are powerful tools both for gene therapeutic applications and basic research in the central nervous system. However, the cellular response after lentiviral transduction needs to be further investigated and evaluated in different species before beginning clinical trials.

ACKNOWLEDGMENTS

We thank M. Michiels and S. Willems for excellent technical assistance. We are grateful to Dr. W. Annaert for help with confocal microscopy, to Dr. H. Demeulemeester for help with stereotactic neurosurgery, and to Dr. F. Vandesande for use of photographic and image analysis equipment. The HIV-1-derived lentivirus vector system was a kind gift from Dr. O. Danos (Evry, France), Dr. D. Trono (University of Geneva, Switzerland), and Dr. P. Charneau (Institut Pasteur, Paris).

V.B. and Z.D. are postdoctoral fellows of the Flemish Fund for Scientific Research (FWO Vlaanderen). This work was funded by IDO grant 98/006 from the KU Leuven Research Council and the STWW program of the Flemish Institute supporting Scientific-Technological Research in Industry (IWT).

REFERENCES

AMADO, R.G., and CHEN, I.S. (1999). Lentiviral vectors—the promise of gene therapy within reach? Science **285**, 674–676.

BAEKELANDT, V., CLAEYS, A., CHEREPANOV, P., DE CLERCQ, E., DE STROOPER, B., NUTTIN, B., and DEBYSER, Z. (2000a). DNA-dependent protein kinase is not required for efficient lentivirus integration. J. Virol. **74**, 11278–11285.

BAEKELANDT, V., DE STROOPER, B., NUTTIN, B., and DEBYSER, Z. (2000b). Gene therapeutic strategies for neurodegenerative diseases. Curr. Opin. Mol. Ther. **2,** 540–554.

BARRY, S.C., HARDER, B., BRZEZINSKI, M., FLINT, L.Y., SEPPEN, J., and OSBORNE, W.R. (2001). Lentivirus vectors encoding both central polypurine tract and posttranscriptional regulatory element provide enhanced transduction and transgene expression. Hum. Gene Ther. **12,** 1103–1108.

BENSADOUN, J.C., DEGLON, N., TSENG, J.L., RIDET, J.L., ZURN, A.D., and AEBISCHER, P. (2000). Lentiviral vectors as a gene delivery system in the mouse midbrain: Cellular and behavioral improvements in a 6-OHDA model of Parkinson's disease using GDNF. Exp. Neurol. **164,** 15–24.

BLÖMER, U., NALDINI, L., KAFRI, T., TRONO, D., VERMA, I.M., and GAGE, F.H. (1997). Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. J. Virol. **71,** 6641–6649.

BLÖMER, U., KAFRI, T., RANDOLPH-MOORE, L., VERMA, I.M., and GAGE, F.H. (1998). Bcl-xL protects adult septal cholinergic neurons from axotomized cell death. Proc. Natl. Acad. Sci. U.S.A. **95,** 2603–2608.

BOSCH, A., PERRET, E., DESMARIS, N., TRONO, D., and HEARD, J.M. (2000). Reversal of pathology in the entire brain of mucopolysaccharidosis type VII mice after lentivirus-mediated gene transfer. Hum. Gene Ther. **11,** 1139–1150.

CHEREPANOV, P., PLUYMERS, W., CLAEYS, A., PROOST, P., DE CLERCQ, E., and DEBYSER, Z. (2000). High-level expression of active HIV-1 integrase from a synthetic gene in human cells. FASEB J. **14,** 1389–1399.

CISTERNI, C., HENDERSON, C.E., AEBISCHER, P., PETTMANN, B., and DEGLON, N. (2000). Efficient gene transfer and expression of biologically active glial cell line-derived neurotrophic factor in rat motoneurons transduced with lentiviral vectors. J. Neurochem. **74,** 1820–1828.

CONSIGLIO, A., QUATTRINI, A., MARTINO, S., BENSADOUN, J.C., DOLCETTA, D., TROJANI, A., BENAGLIA, G., MARCHESINI, S., CESTARI, V., OLIVERIO, A., BORDIGNON, C., and NALDINI, L. (2001). In vivo gene therapy of metachromatic leukodystrophy by lentiviral vectors: Correction of neuropathology and protection against learning impairments in affected mice. Nat. Med. **7,** 310–316.

DEGLON, N., TSENG, J.L., BENSADOUN, J.C., ZURN, A.D., ARSENIJEVIC, Y., PEREIRA DE ALMEIDA, L., ZUFFEREY, R., TRONO, D., and AEBISCHER, P. (2000). Self-inactivating lentiviral vectors with enhanced transgene expression as potential gene transfer system in Parkinson's disease. Hum. Gene Ther. **11,** 179–190.

DULL, T., ZUFFEREY, R., KELLY, M., MANDEL, R.J., NGUYEN, M., TRONO, D., and NALDINI, L. (1998). A third-generation lentivirus vector with a conditional packaging system. J. Virol. **72,** 8463–8471.

FOLLENZI, A., AILLES, L.E., BAKOVIC, S., GEUNA, M., and NALDINI, L. (2000). Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 *pol* sequences. Nat. Genet. **25,** 217–222.

FRANKLIN, K.B.J., and PAXINOS, G. (1997). *The Mouse Brain in Stereotaxic Coordinates.* (Academic Press, San Diego, CA).

HOTTINGER, A.F., AZZOUZ, M., DEGLON, N., AEBISCHER, P., and ZURN, A.D. (2000). Complete and long-term rescue of lesioned adult motoneurons by lentiviral-mediated expression of glial cell line-derived neurotrophic factor in the facial nucleus. J. Neurosci. **20,** 5587–5593.

KAFRI, T., BLÖMER, U., PETERSON, D.A., GAGE, F.H., and VERMA, I.M. (1997). Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat. Genet. **17,** 314–317.

KARPATI, G., LOCHMULLER, H., NALBANTOGLU, J., and DURHAM, H. (1996). The principles of gene therapy for the nervous system. Trends Neurosci. **19,** 49–54.

KIRIK, D., ROSENBLAD, C., BJÖRKLUND, A., and MANDEL, R.J. (2000). Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: Intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. J. Neurosci. **20,** 4686–4700.

KLEIN, R.L., MEYER, E.M., PEEL, A.L., ZOLOTUKHIN, S., MEYERS, C., MUZYCZKA, N., and KING, M.A. (1998). Neuron-specific transduction in the rat septohippocampal or nigrostriatal pathway by recombinant adeno-associated virus vectors. Exp. Neurol. **150,** 183–194.

KORDOWER, J.H., BLOCH, J., MA, S.Y., CHU, Y., PALFI, S., ROITBERG, B.Z., EMBORG, M., HANTRAYE, P., DEGLON, N., and AEBISCHER, P. (1999). Lentiviral gene transfer to the nonhuman primate brain. Exp. Neurol. **160,** 1–16.

KORDOWER, J.H., EMBORG, M.E., BLOCH, J., MA, S.Y., CHU, Y., LEVENTHAL, L., MCBRIDE, J., CHEN, E.Y., PALFI, S., ROITBERG, B.Z., BROWN, W.D., HOLDEN, J.E., PYZALSKI, R., TAYLOR, M.D., CARVEY, P., LING, Z., TRONO, D., HANTRAYE, P., DEGLON, N., and AEBISCHER, P. (2000). Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science **290,** 767–773.

KUHN, H.G., DICKINSON-ANSON, H., and GAGE, F.H. (1996). Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. J. Neurosci. **16,** 2027–2033.

MCCOWN, T.J., XIAO, X., LI, J., BREESE, G.R., and SAMULSKI, R.J. (1996). Differential and persistent expression patterns of CNS gene transfer by an adeno-associated virus (AAV) vector. Brain Res. **713,** 99–107.

MITROPHANOUS, K., YOON, S., ROHLL, J., PATIL, D., WILKES, F., KIM, V., KINGSMAN, S., KINGSMAN, A., and MAZARAKIS, N. (1999). Stable gene transfer to the nervous system using a non-primate lentiviral vector. Gene Ther. **6,** 1808–1818.

MIYOSHI, H., BLÖMER, U., TAKAHASHI, M., GAGE, F.H., and VERMA, I.M. (1998). Development of a self-inactivating lentivirus vector. J. Virol. **72,** 8150–8157.

MOCHIZUKI, H., SCHWARTZ, J.P., TANAKA, K., BRADY, R.O., and REISER, J. (1998). High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. J. Virol. **72,** 8873–8883.

MULLEN, R.J., BUCK, C.R., and SMITH, A.M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. Development **116,** 201–211.

NALDINI, L., BLÖMER, U., GALLAY, P., ORY, D., MULLIGAN, R., GAGE, F.H., VERMA, I.M., and TRONO, D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272,** 263–267.

POESCHLA, E.M., WONG-STAAL, F., and LOONEY, D.J. (1998). Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. Nat. Med. **4,** 354–357.

ROSENBLAD, C., GRONBORG, M., HANSEN, C., BLOM, N., MEYER, M., JOHANSEN, J., DAGO, L., KIRIK, D., PATEL, U.A., LUNDBERG, C., TRONO, D., BJÖRKLUND, A., and JOHANSEN, T.E. (2000). In vivo protection of nigral dopamine neurons by lentiviral gene transfer of the novel GDNF-family member neublastin/artemin. Mol. Cell. Neurosci. **15,** 199–214.

SMITH, R.L., TRAUL, D.L., SCHAACK, J., CLAYTON, G.H., STALEY, K.J., and WILCOX, C.L. (2000). Characterization of promoter function and cell-type-specific expression from viral vectors in the nervous system. J. Virol. **74,** 11254–11261.

STERIO, D.C. (1984). The unbiased estimation of number and sizes of arbitrary particles using the disector. J. Microsc. **134,** 127–136.

TAKAHASHI, M., MIYOSHI, H., VERMA, I.M., and GAGE, F.H. (1999). Rescue from photoreceptor degeneration in the *rd* mouse by

human immunodeficiency virus vector-mediated gene transfer. J. Virol. **73,** 7812–7816.

THEMIS, M., FORBES, S.J., CHAN, L., COOPER, R.G., ETHERIDGE, C.J., MILLER, A.D., HODGSON, H.J., and COUTELLE, C. (1998). Enhanced in vitro and in vivo gene delivery using cationic agent complexed retrovirus vectors. Gene Ther. **5,** 1180–1186.

WHITE, S.M., RENDA, M., NAM, N.Y., KLIMATCHEVA, E., ZHU, Y., FISK, J., HALTERMAN, M., RIMEL, B.J., FEDEROFF, H., PANDYA, S., ROSENBLATT, J.D., and PLANELLES, V. (1999). Lentivirus vectors using human and simian immunodeficiency virus elements. J. Virol. **73,** 2832–2840.

YOUNG, D., LAWLOR, P.A., LEONE, P., DRAGUNOW, M., and DURING, M.J. (1999). Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat. Med. **5,** 448–453.

ZENNOU, V., PETIT, C., GUETARD, D., NERHBASS, U., MONTAGNIER, L., and CHARNEAU, P. (2000). HIV-1 genome nuclear import is mediated by a central DNA flap. Cell **101,** 173–185.

ZENNOU, V., SERGUERA, C., SARKIS, C., COLIN, P., PERRET, E., MALLET, J., and CHARNEAU, P. (2001). The HIV-1 DNA flap stimulates HIV vector-mediated cell transduction in the brain. Nat. Biotechnol. **19,** 446–450.

ZUFFEREY, R., NAGY, D., MANDEL, R.J., NALDINI, L., and TRONO, D. (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat. Biotechnol. **15,** 871–875.

ZUFFEREY, R., DULL, T., MANDEL, R.J., BUKOVSKY, A., QUIROZ, D., NALDINI, L., and TRONO, D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J. Virol. **72,** 9873–9880.

ZUFFEREY, R., DONELLO, J.E., TRONO, D., and HOPE, T.J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J. Virol. **73,** 2886–2892.

Address reprint requests to:
Dr. Veerle Baekelandt
Provisorium 1
Minderbroedersstraat 17
B-3000 Leuven, Belgium

E-mail: veerle.baekelandt@med.kuleuven.ac.be

Received for publication March 20, 2001; accepted after revision March 12, 2002.

Published online: April 3, 2002.